

Protection by nitrite against the ischemic effects induced by acute myocardial infarction in mice

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ABSTRACT

Objective: This research was aimed to investigate the correct dose of nitrite that would act as a protection against the ischemic effects induced by acute myocardial infarction (AMI).

Methods: Mice were randomly divided into a sham-operation group (sham), an AMI operation group (AMI), and a nitrite pretreatment+AMI operation group (N+AMI). Seven days before the AMI operation, mice in the N+AMI group were pretreated with sodium nitrite in drinking water.

Results: One week after the AMI operation, serum lactate dehydrogenase (LDH) and creatine kinase (CK) activities in both AMI and N+AMI group were significantly higher than those in the sham group, but there were no significant differences between AMI and N+AMI mice. Contents of inducible nitric oxide synthase (iNOS) in the noninfarct area of the left ventricle in the N+AMI mice were significantly higher than those in the AMI mice, with no difference in the infarct area. Coagulation necrosis in the cardiomyocytes was observed in both AMI and N+AMI mice; however, it was less severe in the N+AMI mice. Western blot analyses showed that nitrite pretreatment resulted in up-regulation of antiapoptotic factors Bcl-2 and p21waf1/cip1 signal proteins, but down-regulation of the proapoptotic factor Bax signal protein. Furthermore, nitrite pretreatment also showed significant alleviation of AMI-induced signal protein expressions of inflammatory factors of NF- κ B and oxidative factors of Hsp 70 and HO-1.

Conclusion: These results suggest that nitrite show certain protective effects against the ischemic effects induced by AMI in mice, which might be attributed to the synthesis of NO induced by iNOS through up-regulation of antiapoptotic factors and down-regulation of proapoptotic and inflammatory factors. (*Anatol J Cardiol* 2017; 18: 315-20)

Keywords: nitrite, acute myocardial infarction, ischemia, inducible nitric oxide synthase, cell signaling

Introduction

Nitrite is an inactive terminal oxygenated metabolite of endogenous nitric oxide (NO) (1). Under a hypoxic–acidic environment in the body, nitrite can be deoxidized to NO by internal nitrite reductases. NO is an important biological regulator and is a fundamental component in the fields of neuroscience, physiology, and immunology (2). NO achieves its biological effect by activating guanylate cyclase and subsequently increasing cyclic guanosine monophosphate (cGMP), which is known as the L-arginine/nitric oxide (L-Arg/NO) pathway (3). Nitric oxide synthase (NOS) is the rate-limiting enzyme of NO synthesis (4), which is the most important step in regulating the NO level. NOS can be divided into two types, constitutive NOS (cNOS) and

inducible NOS (iNOS) (5). iNOS regulates NO synthesis at the expression, transcription, and translation levels. Recent studies have demonstrated that nitrite can protect the ischemic tissues against reperfusion injury (6). Ischemia–reperfusion (I/R) may bring about the formation of oxygen radicals, followed by widespread lipid and protein oxidative modifications, apoptosis, and necrosis in ischemic tissues (7); however, NO in low concentrations may reverse such conditions (8). Other studies have also suggested that myoglobin as a functional nitrite reductase that regulates NO generation and controls mitochondrial electron transfer limits the generation of reactive oxygen species and confirms a cytoprotective response to I/R injury (9,10). Therefore, nitrite may emerge as an endogenous signaling molecule with potential therapeutic implications for cardiovascular diseases (11).

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Accepted Date: 03.08.2017 **Available Online Date:** 10.11.2017

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DOI:10.14744/AnatolJCardiol.2017.7952



In this study, mice were pretreated with/without sodium nitrite in drinking water for 7 days. On the 7th day after AMI operation, surviving animals were sacrificed. Blood serum, myocardium, and organ tissues were collected. Protection by and the underlying cell signal mechanism of nitrite against the ischemic effects were investigated.

Methods

Animals and materials

C57BL/6 male mice were purchased from an experimental animal company. XTL continuous zoom stereomicroscope (Shenzhen Ruiwode Life Technology Company, Shenzhen, China) and MicroVent 1 small animal ventilator (Pittsfield, USA) were used in the microsurgical operations. Hitachi 7600-110 autoanalyzer was used for biochemical analyses. Chloral hydrate and isoflurane were obtained from Sun Chemical Technology (Shanghai, China). In addition, microsurgical instruments, endotracheal intubations, and disposable intravenous catheters (22 G) were used in this study. The iNOS assay kit was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Thirty C57BL/6 male mice were randomly divided into three groups ($n=10$ mice/group): a sham-operation group (sham), an AMI operation group (AMI), and a nitrite pretreatment+AMI operation group (N+AMI). Before the AMI operation, mice in the N+AMI group were pretreated with sodium nitrite in drinking water (50 mg/L in double distilled water) for 7 days according to previous studies (11,12). Mice were housed in controlled temperature, humidity, and 12-h light–dark cycle with free access to chow and water. Mice studies were approved by the Ningbo University Institutional Animal Care and Use Committee.

Establishment of an AMI model

Under 7% chloral hydrate anesthesia (250 mg/kg, intraperitoneal injection), the mouse was placed on an operating platform in the right lateral position. Then, the mouse was connected to a MicroVent 1 small animal ventilator by intubation to control breathing. The tidal volume of the ventilator was adjusted to 4–5 mL/min, and the respiratory frequency was set at 130–140/min. Fixed in the right lateral position, the mouse was incised at the left fifth intercostal space. Then, the thoracic cavity was opened and the heart was exposed. To induce AMI, after the left coronary artery was identified, it was ligated with a 7/0 no-damage silk suture. Then, the thorax was stitched and closed layer by layer. After removing the ventilator, tweezers were used to stimulate the foot of the mouse to promote spontaneous breathing. Five or 10 min later, endotracheal intubation could be pulled out when the spontaneous breathing returned.

The left coronary artery of all 20 mice in the AMI and N+AMI group was ligated to establish an AMI model. All operations were similarly performed for all groups, except for the sham group, in which there was no ligation of the left coronary artery. After the operation, similar living conditions were maintained for all surviving animals.

Biochemical and histopathological analyses

On the 7th day after surgery, mice were weighed and anesthetized with inhaled 2% isoflurane. After anesthesia, blood samples were collected via the femoral artery. Serum was obtained by centrifugation at 3500 rpm for 10 min and stored at -80°C until use. Heart tissues were obtained as quickly as possible and were rinsed in saline. After weighing, left ventricular tissues obtained from the AMI and N+AMI groups was separated as infarct area tissue and noninfarct area tissue and tissue homogenates were prepared according to the method by Sindler et al. (13, 14). The homogenates were stored at -80°C for the measurement of iNOS activity. After organ weighing, heart tissues were fixed in a 10% formalin solution for 24 h, embedded in paraffin, and sliced. Tissue sections were stained with H&E and observed under the microscope. LDH and CK activity was measured using an automatic biochemical analyzer. The iNOS activity was detected following the manufacturer's instructions.

Western blot analysis

Heart tissues were stored at -80°C until used, as described previously. About 100 mg heart tissue was homogenized using glass homogenizers in 1 mL NP40 lysis buffer (Beyotime, Shanghai, China) with 1 mM phenylmethylsulfonyl fluoride (PMSF) (Beyotime, Shanghai, China) and then slightly shaken for 40 min. During experiments, the protein sample was always kept on ice. After centrifugation (12,000 rpm for 20 min at 4°C), the supernatant of the samples was collected and total protein was measured. The total protein was mixed with the loading buffer (Beyotime, Shanghai, China) and heated in boiling water for 5 min. SDS-PAGE polyacrylamide gel (10% or 12%) electrophoresis was performed and transferred to 0.45 μm PVDF membrane (Boston, USA) using an electrophoresis and blotting system (CA, USA). The substrate was blocked using 5% nonfat milk and incubated with primary and then secondary antibodies. The specific antibodies against Bax, Bcl-2, and p21waf1/cip1 (200 $\mu\text{g}/\text{mL}$, 1: 1000 dilution) were from Cell Signaling Technology (Massachusetts, the USA). The specific antibodies against NF- κ B, heat shock protein 70 (Hsp 70) and heme oxygenase-1 (HO-1) (200 $\mu\text{g}/\text{mL}$, 1: 1000 dilution) were from Santa Cruz Biotechnology (Santa Cruz, CA, the USA). The specific antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (100 $\mu\text{g}/\mu\text{L}$, 1: 500 dilution) were from Shanghai. The band density was analyzed using the Quantity One software (Bio-Rad, CA, the USA).

Ethics approval

This in vivo study has been examined and approved by the experimental animal Ethics Committee. There was no violation of experimental animal ethics principle in this study.

Statistical methods

Kolmogorov–Smirnov test was used for normality. Data in normal distribution were expressed as $\bar{x}\pm s$. Data in skewed distribution were expressed as medians (Q1, Q3). Comparison be-

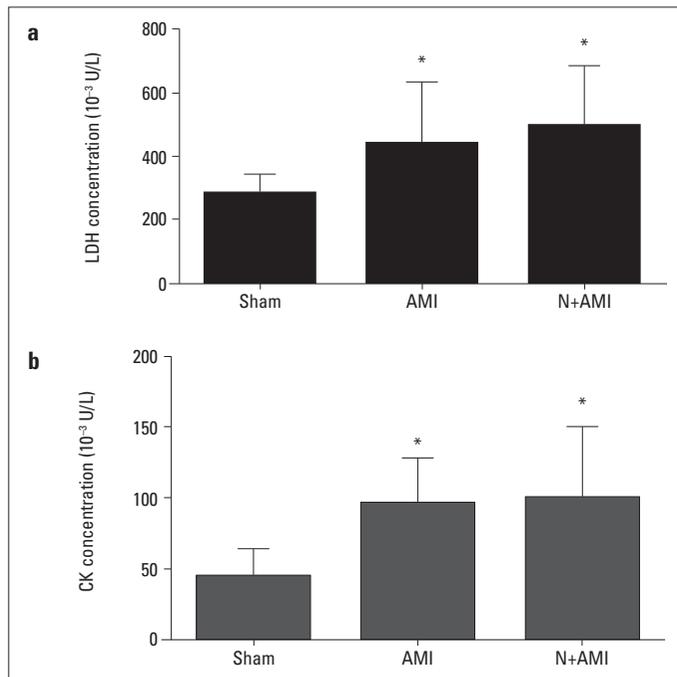


Figure 1. Serum LDH and CK activity (10⁻³ U/L)
 *Significantly different compared with the sham group (p<0.05). Serum LDH (A) and CK (B) activities in both AMI and N+AMI groups were significantly enhanced compared with the sham group. However, no significant difference was found between AMI and N+AMI groups in both LDH and CK activities.

tween two groups and three groups were analyzed by t-test and one-way ANOVA for normal distributed data. The Kruskal–Wallis H test was used for three groups, and for multiple comparisons between groups, Wilcoxon rank sum test was applied, but the possibility of type error (α) was adjusted, $\alpha=0.017$. All statistical analyses were performed using SPSS 13.0 statistical software. A p-value of <0.05 was considered significant.

Results

Animal survival, mortality, change of animal body weight after surgery

Data from a total of 30 mice were analyzed. In the sham group, three out of 10 mice died due to an anesthesia overdose. In the AMI and N+AMI groups, six out of 20 mice died after the opera-

Table 1. Comparison of iNOS activity in myocardium

Test area	Experimental groups	Mice number	iNOS (U/mL) (Median, Q1, Q3)
Non-infarct area	Sham	4	0.4600 (0.4543, 0.4812)
	AMI	4	0.0500 (0.0274, 0.0721)
	N+AMI	5	0.1100 (0.1098, 0.1121)
	AMI+(N+AMI)	9	0.0899 (0.0543, 0.1100)*
Infarct area	AMI	4	0.0360 (0.0343, 0.0558)
	N+AMI	5	0.0760 (0.0628, 0.0879)

AMI-acute myocardial infarction; iNOS-inducible NOS; N+AMI-nitrite pretreatment+AMI operation. *P<0.05, significant differences versus the sham group.

tion. Of these, two died of anesthesia overdose on the operating platform and four died 2–4 days after the operation. An autopsy of these four mice revealed that one death was due to pleural effusion and three due to cardiac rupture. Twenty-one mice survived after surgery.

The mean body weight of the mice was 29.00±3.24 g (30 mice in total) before surgery and 24.78±2.40 g 7 days after surgery (21 mice in total) (t=4.45, p<0.05), which suggested that surgical trauma could result in a reduction in the body weight of the mice. The reduction in body weight (2.57±1.90 g) in the N+AMI group was significantly less than that in the AMI group (5.14±1.95 g) (p<0.05).

Heart weight/body weight coefficients

According to an autopsy, no myocardial infarction was found in the hearts of the sham mice, but different degrees of myocardial infarction were found in those of AMI and N+AMI mice. The heart weight/body weight coefficients were 5.15±0.80 (10⁻³ g/g) in sham mice and 6.52±1.13 (10⁻³ g/g) in AMI and N+AMI mice. There was a significant difference between the coefficients of sham mice and ligated mice (AMI and N+AMI groups) (t=-2.25, p<0.05).

Changes of serum LDH and CK activity and the content of iNOS in myocardium

Serum LDH and CK activities in AMI and N+AMI mice were significantly increased compared with those in the sham mice, but no difference was found between AMI and N+AMI mice (Fig.

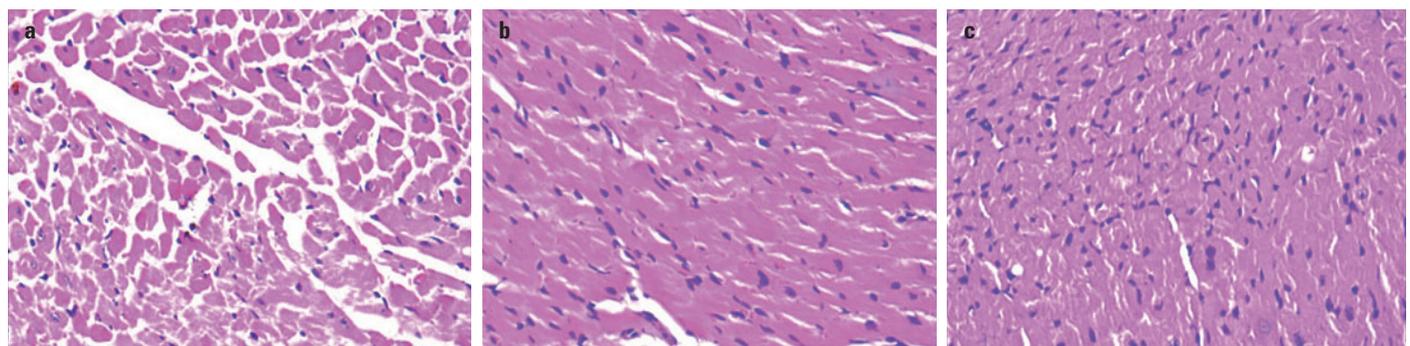


Figure 2. Histopathological examinations of myocardial tissue (a) Myocardial tissue in sham mice; (b) Myocardial tissue in AMI mice; (c) Myocardial tissue in N+AMI mice (x400 magnification).

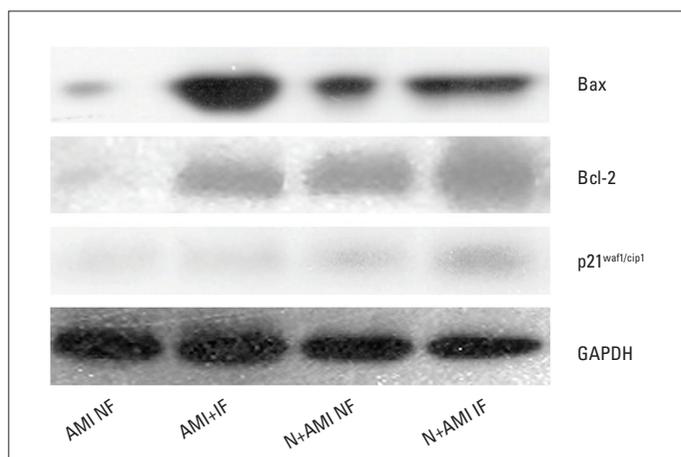


Figure 3. Effect of nitrite pretreatment on the signal protein expressions of Bax, Bcl-2, and p21 waf1/cip1 in the myocardium of AMI mice
AMI NF, noninfarct myocardium of AMI mice; AMI IF, infarct myocardium of AMI mice; N+AMI NF, noninfarct myocardium of N+AMI mice; N+AMI IF, infarct myocardium of N+AMI mice.

1). The content of iNOS (Table 1) in the noninfarct area of AMI and N+AMI mice was significantly decreased compared with that in the whole left ventricle of sham mice ($p < 0.05$). Compared with the AMI group, the iNOS content in the N+AMI group showed an obvious increased tendency in both infarct and noninfarct areas.

Histopathological examination

In sham mice, myocardial cells showed no pathological changes, with a smooth arrangement and clear organizational structure (Fig. 2A). In the left coronary artery of the ligated mice of AMI and N+AMI groups, scattered coagulation necrosis of myocardial cells was found around the coronary blood supply area, showing increased eosinophilic cytoplasm and unclear nuclear and organizational structures, as well as thin and stretched myocardial cells. These pathological changes in N+AMI mice appeared less severe than those in AMI mice (Fig. 2B and C, respectively).

Determination of myocardial Bax, Bcl-2, p21waf1/cip1, Hsp 70, NF- κ B, and HO-1 signal protein expressions by Western blot

Both Bax and Bcl-2 protein expressions in the infarct myocardium of AMI mice were up-regulated, and nitrite pretreatment could alleviate only the elevated Bax protein expression. N+AMI mice showed a significant up-regulation of Bcl-2 and p21waf1/cip1 protein expression in both infarct and noninfarct area of myocardium (Fig. 3).

Significant up-regulation expressions of Hsp 70, NF- κ B, and HO-1 were found in the infarct area, but not in the noninfarct area, of AMI mice. Nitrite pretreatment showed certain inhibition of the up-regulation expressions of Hsp 70, NF- κ B, and HO-1 (Fig. 4).

Discussion

In the present study, to detect the protection by nitrite against the ischemic effects induced by acute myocardial in-

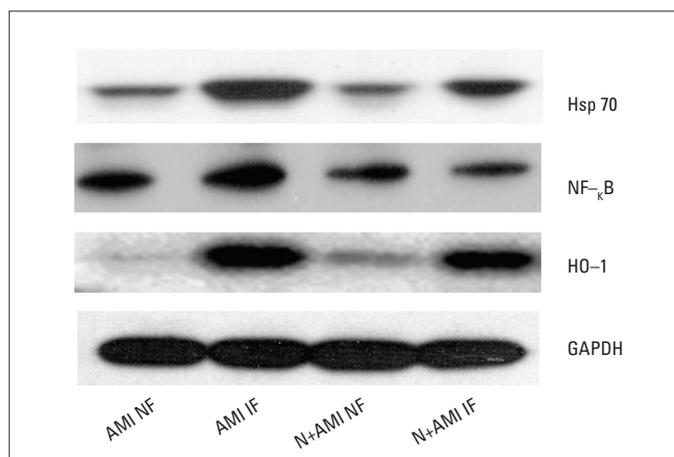


Figure 4. Effect of nitrite pretreatment on Hsp 70, NF- κ B, and HO-1 in the myocardium of AMI mice

AMI NF, noninfarct myocardium of AMI mice; AMI IF, infarct myocardium of AMI mice; N+AMI NF, noninfarct myocardium of N+AMI mice; N+AMI IF, infarct myocardium of N+AMI mice.

fraction, we pretreated the mice with sodium nitrite in drinking water (50 mg/L) for 7 days before the AMI operation. We found that nitrite has certain protective effects against the ischemic effects induced by AMI in mice, which might be attributed to the synthesis of NO induced by iNOS through up-regulation of antiapoptotic factors and down-regulation of proapoptotic and inflammatory factors.

NO is a powerful vasodilator with a short half-life of a few seconds in the blood (15) and is an important cellular signaling molecule involved in many physiological and pathological processes (16). The production of NO in the body is catalyzed by a family of enzymes called NOSs (17). Nitrite, as a key storage of NO in blood and tissue, can be reduced to NO under certain conditions, such as a decrease in NO due to ischemia or anoxia.

Recently, nitrite-derived NO has been reported to exert a profound protection against brain, liver, and heart I/F injuries. However, the mechanisms are unclear (18). Johnson et al. (19) intravenously infused NaNO₂ (12.5–50 mmol/kg/h) starting 30 min post occlusion followed by reperfusion 1 h later in cats subjected to myocardial infarction by occlusion of the left anterior descending coronary artery. They concluded that NaNO₂ exerted a significant protective action during ischemia and reperfusion injury. Webb et al. (20) reported that pretreatment of nitrite (10 and 100 μ M) before ischemia reduced the infarct size and improved the recovery of left ventricular function by using an isolated Langendorff rat heart. This suggests that the supplement of exogenous nitrite can limit the myocardial I/F injury. Another study demonstrated that nitrite could protect the ischemic myocardium at the beginning of reperfusion and that the pretreatment of nitrite to myocardium could be performed systemically or orally (21). Our results showed that AMI surgery can result in a significant decrease in the mouse's body weight. The coronary artery ligation can cause a significant enlargement of the heart demonstrated by an increase of the heart weight/body

weight coefficients. Biochemical analyses showed that an AMI operation can significantly increase the serum LDH and CK activity, but no difference was found between sodium nitrite pretreated mice and no sodium nitrite pretreated AMI mice. This suggests that sodium nitrite pretreatment cannot significantly alleviate the release of LDH and CK enzymes from the ischemic myocardial cells induced by the AMI operation. However, the content of iNOS in the noninfarct area of the left ventricle in sodium nitrite pretreated AMI mice was significantly higher than that in no sodium nitrite pretreated AMI mice, which indicates that sodium nitrite pretreatment may elevate the NO level in the noninfarct area, consistent with the results of previous studies. Coagulation necrosis in the myocardial cells was observed in both AMI and sodium nitrite pretreated AMI mice, but less severe in sodium nitrite pretreated AMI mice.

Bcl-2 and Bax are two of the BCL-2 family proteins (22), which are important parts in the formation of apoptotic pores in the outer mitochondrial membrane (23). Bcl-2, as an antiapoptotic protein, can inhibit the apoptotic pore formation, whereas Bax is a proapoptotic protein that promotes this formation when activated (23). The p21/waf1 protein, also known as cyclin-dependent kinase inhibitor 1, regulates the cell cycle progression. Many studies have shown that p21 has an important part in the NO regulation to inhibit smooth muscle cell proliferation (24). In addition, Israsen et al. (25) reported that p21/waf1 protein mediates the proapoptotic effects of BMP4 on ventricular zone progenitor cells. Our study shows that the nitrite pretreatment can alleviate the apoptosis of myocardium by up-regulating Bcl-2 and p21/waf1/cip1 proteins, which may be useful in protecting the myocardium against AMI-induced ischemia.

Hsp 70 is an important regulatory part of cells for protein folding and helps to defend against thermal or oxidative stress (26). When the stress is eliminated, cells go into a recovery phase and the Hsp 70 level decreases (26). In our study, the expression of Hsp 70 remained in the mice 1 week after AMI. The lower Hsp 70 level in the noninfarct myocardial area of N+AMI mice may show indirect evidence of less damage and better protection. The HO-1 level was downregulated by nitrite pretreatment, which is consistent with previous studies (27).

The activation of NF- κ B can also stimulate numerous genes and indirectly mediate the adherence and aggregation of neutrophils. The down-regulation of NF- κ B expression thus improved the coronary microvasculature and myocardium inflammation (28). Our results suggest that these important transcription factors may be involved in the nitrite effect in oxidative and nitrosative signaling.

Study limitations

Our study indicates the protective effects of nitrite against ischemic injury at low doses and reveals its molecular mechanism, which shows a potential for treating AMI-induced ische-

mic damage. It is worthy to note that although nitrite at low doses shows certain protective effects against ischemic damage, high nitrite doses may be toxic to the human body.

Conclusion

In summary, our results indicate that nitrite has certain protective effects against AMI-induced ischemic injury. The protective effects of nitrite against ischemic injury may be attributed to the increase in NO levels induced by iNOS and the involvement of multiple signaling pathways, including antiapoptotic and proapoptotic, as well as inflammatory factors. Our findings may provide further support to investigate the proper dose of nitrite for the treatment of ischemic injury.

Disclosure statement

The authors declare that they have no competing interests.

Acknowledgments

The authors would like to thank Ms. Linda Bowman (from Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA) for her assistance in the preparation of this article. This work was partly supported by the National Nature Science Foundation of China (Grant No 81273111), the Scientific Projects of Zhejiang Province (2015C33148 and 2015C37117), the Foundations of Innovative Research Team of Educational Commission of Zhejiang Province (T200907), Ningbo Scientific Innovation Team for Environmental Hazardous Factor Control and Prevention (2016C51001), Zhejiang Key Laboratory of Pathophysiology (201703), and KC Wong Magna Fund in Ningbo University.

Conflict of interest

This research was supported by the Scientific Research Projects Fund of Namik Kemal University (NKUBAP02.YL.16.046).

Peer-review: Externally peer-reviewed.

Authorship contributions: Concept – Y.W., X.S., X.Y.; Design – Y.W., X.S., X.Y.; Supervision – J.Z., X.L.; Fundings – L.B., M.D., B.Z., J.Z.; Materials – L.B., M.D.; Data collection &/or processing – Y.W., X.S., J.Z.; Analysis &/or interpretation – Y.W., X.Y., H.S., Y.G.; Literature search – X.Y., H.S., Y.G.; Writing – Y.W., X.S., X.L.; Critical review – H.S., Y.G., X.L., L.B., M.D., B.Z.

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